Lipopolysaccharide enhances apoptosis of corpus luteum in isolated perfused bovine ovariesin vitro

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Abstract: Lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria, has detrimental effects on structure and function of the bovine corpus luteum (CL) in vivo. The objective was to investigate whether these effects were mediated directly by LPS or via LPS-induced release of PGF2 . Bovine ovaries with a mid-cycle CL were collected immediately after slaughter and isolated perfused for 240 min. After 60 min of equilibration, LPS (0.5 g/mL) was added to the medium of five ovaries, whereas an additional six ovaries were not treated with LPS (control). After 210 min of perfusion, all ovaries were treated with 500 iu of hCG. In the effluent perfusate, concentrations of progesterone (P4) and PGF2 were measured every 10 and 30 min, respectively. Punch biopsies of the CL were collected every 60 min and used for RT-qPCR to evaluate mRNA expression of receptors for LPS (TLR2, -4) and LH (LHCGR), the cytokine TNFA, steroidogenic (STAR, HSD3B), angiogenic (VEGFA121, FGF2) and vasoactive (EDN1) factors, and factors of prostaglandin synthesis (PGES, PGFS, PTGFR) and apoptosis (CASP3, -8, -9). Treatment with LPS abolished the hCG-induced increase in P4 (P 0.05); however, there was only a tendency (P=0.10) for increased release of PGF2 at 70 min after LPS challenge. Furthermore, mRNA abundance of TLR2, TNFA, CASP3, CASP8, PGES, PGFS, and VEGFA121 increased (P 0.05) after LPS treatment, whereas all other factors remained unchanged (P>0.05). In conclusion, reduced P4 responsiveness to hCG in LPS-treated ovaries in vitro was not due to reduced steroidogenesis, but was attributed to enhanced apoptosis. However, an impact of luteal PGF2 could not be excluded.

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Lipopolysaccharide enhances apoptosis of corpus luteum in isolated perfused bovine ovaries *in vitro*

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Short title: LPS-effects on CL in isolated perfused ovaries

Abstract

Lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria, has detrimental effects on structure and function of the bovine corpus luteum (CL) *in vivo*. The objective was to investigate whether these effects were mediated directly by LPS or *via* LPS-induced release of $PGF_{2\alpha}$. Bovine ovaries with a mid-cycle CL were collected immediately after slaughter and isolated perfused for 240 min. After 60 min of equilibration, LPS (0.5 µg/mL) was added to the medium of five ovaries, whereas an additional six ovaries were not treated with LPS (control). After 210 min of perfusion, all ovaries were treated with 500 iu of hCG. In the effluent perfusate, concentrations of progesterone (P_4) and $PGF_{2\alpha}$ were measured every 10 and 30 min, respectively. Punch biopsies of the CL were collected every 60 min and used for RT-qPCR to evaluate mRNA expression of receptors for LPS (*TLR2*, *-4*) and LH (*LHCGR*), the cytokine *TNFA*, steroidogenic (*STAR*, *HSD3B*), angiogenic (*$VEGFA_{121}$*, *FGF2*) and vasoactive (*EDN1*) factors, and factors of prostaglandin synthesis (*PGES*, *PGFS*, *PTGFR*) and apoptosis (*CASP3*, *-8*, *-9*). Treatment with LPS abolished the hCG-induced increase in P_4 ($P \leq 0.05$); however, there was only a tendency ($P=0.10$) for increased release of $PGF_{2\alpha}$ at 70 min after LPS challenge. Furthermore, mRNA abundance of *TLR2*, *TNFA*, *CASP3*, *CASP8*, *PGES*, *PGFS*, and *$VEGFA_{121}$* increased ($P \leq 0.05$) after LPS treatment, whereas all other factors remained unchanged ($P>0.05$). In conclusion, reduced P_4 responsiveness to hCG in LPS-treated ovaries *in vitro* was not due to reduced steroidogenesis, but was attributed to enhanced apoptosis. However, an impact of luteal $PGF_{2\alpha}$ could not be excluded.

Key words: corpus luteum, inflammation, endotoxin, isolated perfused ovary, cattle

1. Introduction

Fertility in dairy cows has decreased for more than a half century (Walsh *et al.* 2011), due in part to inflammatory diseases (Barker *et al.* 1998, LeBlanc *et al.* 2002). Cows with endometritis have prolonged calving-to-conception intervals (CCI) and are replaced sooner, due to reproductive failure (LeBlanc *et al.* 2002). Furthermore, inflammation in non-genital tissues (e.g. mastitis) also prolonged CCI and increased the number of services per conception (Barker *et al.* 1998), attributed in part to activation of immune cells by bacterial cell wall components and production of cytokines (Hansen *et al.* 2004).

In previous studies (Suzuki *et al.* 2001, Herzog *et al.* 2012), lipopolysaccharide (LPS), the endotoxin from the outer membrane of gram-negative bacteria, was used as a model to determine effects of inflammation on fertility in cows. Binding of LPS to toll-like receptor (TLR) 4 (Akira 2003) activates macrophages and triggers cytokine production. Furthermore, instillation of LPS in the mammary gland consistently increases systemic concentrations of cytokine tumor necrosis factor alpha (TNFA; Blum *et al.* 2000). In addition, exogenous LPS reduced fertility due to impairment of ovarian follicular development and ovulation (Suzuki *et al.* 2001, Lavon *et al.* 2008, Williams *et al.* 2008). In cows given LPS during proestrus, the LH peak was significantly retarded or completely inhibited (Suzuki *et al.* 2001). Furthermore, LPS affected morphology and function of the bovine CL. In that regard, intravenous LPS decreased blood progesterone (P_4) concentrations (Giri *et al.* 1991, Herzog *et al.* 2012) and caused temporary reductions in both luteal size and blood flow (Herzog *et al.* 2012). Since $PGF_{2\alpha}$ metabolite concentrations were increased after LPS treatment (Herzog *et al.* 2012), which was consistent with increased $PGF_{2\alpha}$ release from the endometrium during inflammation (Giri *et al.* 1991, Miyamoto *et al.* 2000, Skarzynski *et al.* 2000), it was speculated that enhanced uterine $PGF_{2\alpha}$ induced premature luteolysis. It was noteworthy that TNFA consistently stimulated $PGF_{2\alpha}$ synthesis in endometrial cells during all stages of the

estrous cycle (Miyamoto *et al.* 2000). However, a more recent study (Mishra & Dhali 2007) suggested that LPS might impair the CL, independent of endometrium-derived $PGF_{2\alpha}$. In that study, which used a luteal monolayer culture, LPS induced oxidative stress and decreased cell viability, although an influence of endometrium-derived $PGF_{2\alpha}$ was excluded.

In ruminants, the *in vitro* model of an isolated perfused ovary (IPO) was introduced several decades ago (Romanoff & Pincus 1962, Mills & Morrissette 1970, Stahler & Huch 1971, Sturm & Stahler 1971, Janson *et al.* 1978), mainly to investigate ovarian steroid biosynthesis and energy metabolism. For studying mechanisms of LPS action on the bovine CL, with special consideration of the impact of uterine-derived $PGF_{2\alpha}$, this model has important advantages. At first, compared to *in vivo* studies, the IPO model facilitates determining LPS effects on the CL, excluding any influence of $PGF_{2\alpha}$ from extra-luteal sources. Therefore, this model should be a good alternative to LPS, which has severe systemic effects on cows (Giri *et al.* 1990, Giri *et al.* 1991, Herzog *et al.* 2012). Secondly, compared to cell cultures, the IPO model enables interactions between various cell types (e.g. luteal, vascular, and stromal cells) of the bovine CL without changing their composition and arrangement, including an intact three-dimensional structure and intercellular communication (Brannstrom & Flaherty 1995). Furthermore, the structural integrity of the CL within the surrounding ovarian tissue was maintained in IPO (Stahler & Huch 1971) compared to tissue culture systems.

Using the IPO technique, determination of P_4 and $PGF_{2\alpha}$ in the effluent perfusate enables direct assessment of release of these hormones from the CL. Progesterone synthesis is the most important indicator of functional integrity of the CL (Rekawiecki *et al.* 2008). In that regard, increased P_4 synthesis following LH treatment is evidence of CL responsiveness (Skarzynski *et al.* 2008). Prostaglandin $F_{2\alpha}$ is the most effective mediator of luteolysis in ruminants (McCracken *et al.* 1999). During physiologic luteolysis in cattle, $PGF_{2\alpha}$ is released from the endometrium as well as the CL (Shirasuna *et al.* 2004). Furthermore, gene

99 expression of steroidogenic, angiogenic and vasoactive factors in luteal tissue provided
100 relevant information regarding functionality of the bovine CL (Miyamoto *et al.* 2009,
101 Shirasuna *et al.* 2010). The proinflammatory cytokine TNFA is present in bovine luteal cells,
102 as well as in immune cells (mainly macrophages; Sakumoto *et al.* 2011), and is capable of
103 reducing P_4 secretion, increasing $PGF_{2\alpha}$ production and inducing apoptosis in luteal cell
104 cultures (Okuda & Sakumoto 2003, Skarzynski *et al.* 2005).
105 In this study, we hypothesized that LPS directly suppresses CL function *via* enhanced
106 apoptosis. Thus, the isolated perfused ovary model was established, and the impact of LPS
107 challenge using this *in vitro* system was evaluated in detail.
108

2. Materials and Methods

2.1. Ovaries

Ovaries with *mesovarium* were harvested from the carcasses of clinically healthy cows (*Bos taurus*; including Holstein Friesian, Red Holstein, Swiss Fleckvieh and Brown Swiss) that were slaughtered at a commercial abattoir. Sixteen ovaries containing a CL with an estimated diameter of >20 mm (subsequently confirmed as a mid-cycle CL) and intact *tunica albuginea* as well as *mesovarium* with ovarian vessels were used.

2.2. Preparation of ovaries

Immediately after the ovary was recovered, the *ramus uterinus* and all branches of the ovarian artery with a similar diameter were ligated (Polysorb 0®; Corvidien, Dublin, Ireland). Since the ovarian artery splits into small vessels and forms a convolute that surrounds the ovarian vein before entering the ovary, catheterization was performed proximal to the location where the *ramus uterinus* branches from the ovarian artery (Fig. 1). The ovarian artery was bluntly dissected from the connective tissue, stretched, cut diagonally and enlarged by catheterization with peripheral venous catheters (Terumo Surflow®; Terumo Europe, Leuven, Belgium) using increasing diameters (20 and 18 gauge) until a permanent 16 gauge venous catheter (Vygonüle T; Vygon, Écouen, France) could be installed. The catheter was fixed within the ovarian artery with two circular ligatures (Polysorb 0®).

To avoid coagulation of blood within the vessels, ovaries were flushed with chilled (4 °C), heparinized (Heparin Bichsel 5000 IE/ml®; Bichsel AG, Interlaken, Switzerland; 150 iu heparin/mL medium) Tyrode's solution, containing 136 mmol/L NaCL, 11.9 mmol/L $NaHCO_3$, 5.5 mmol/L D(+)glucose * H_2O, 2 mmol/L KCL, 1.8 mmol/L $CaCL_2$ * $2H_2O$,

1.05 mmol/L $MgCL_2$ * $6H_2O$, 0.416 mmol/L NaH_2PO_4, and 6% (w/v) dextran 70 000 added to distilled water to a volume of 1 L. Flushing was continued until the effluent perfusate was macroscopically clear and the color of the CL changed from red to yellow. Flushed ovaries were transported to the laboratory in ice-chilled heparinized Tyrode's solution.

In the laboratory, ovaries with a vascular pedicle were weighed with a precision balance (Mettler PM 400®; Mettler-Toledo, Greifensee, Switzerland), and average diameter of the CL was determined with ultrasonography (Aquila Esaote Pie Medical; Esaote Biomedica, Cologne, Germany). Only ovaries with a luteal diameter >20 mm were used. Ovaries were fixed with two sutures (Polysorb 0®) at their *extremitas tubaria* and *uterina* on a bipod (Fig. 2). Gravity induced the *margo liber* of the ovary to point upwards and the *margo mesovaricus* to point downwards, with the free ends of the ovarian artery (catheterized) and vein (without catheter) positioned directly above a funnel.

2.3. Isolated perfusion of ovaries

Ovaries (fixed on the bipod) were put in an incubator for newborn humans (Atom Infant Incubator V-850; Atom Medical Corporation, Tokyo, Japan) to ensure perfusion under standardized microclimatic conditions (temperature, 37.5 to 38.5 °C; relative humidity, 75 to 85%). A schematic illustration of the complete set-up is shown (Figure 3).

As medium for the isolated perfusion of the ovary, a modified Tyrode's solution was used (for detailed composition see Section 2.2.). Osmotically active dextran 70 000 was added to the original formula of Tyrode's solution to reduce intercellular edema in the ovary, as suggested (Janson *et al.* 1978, Bjersing *et al.* 1981). Tyrode's solution was oxygenated with carbogen (Oxycarbon medizinal; PanGas, Dagmersellen, Switzerland) using a hollow fiber type oxygenator (Membrana Oxyphan PP50/200; Membrana GmbH, Wuppertal, Germany). To ensure adequate oxygenation, pH and partial pressures of oxygen and carbon dioxide in

the perfusion medium before and after its passage through the ovary were measured 10 min after the start of perfusion, and then every 30 min until the end of perfusion using a blood-gas analysis system (Rapidlap 248 TM, Siemens, Munich, Germany). According to the results of blood-gas analysis, the supply of carbogen and the flow rate were adjusted to sustain a physiologic pH (target value, 7.40). Temperature of the perfusion medium was controlled with a Liebig condenser (GB Kühler Liebig, NSK+H14/23 120mm; UZH Glassblowing Factory, Zurich, Switzerland) connected to a tempered water circulation (Type SSB4; Grant Instruments, Cambridge, England). Directly before entering the ovarian artery, temperature of Tyrode's solution was measured using a thermometer (WDT, Garbsen, Germany), every 5 min between 0 and 20 min after the start of perfusion, and then every 10 min to the end of perfusion. According to these measurements, temperature of the water circulation was adjusted to maintain the Tyrode's solution between 37 and 38 °C, as reported in similar studies (Koos *et al.* 1984, Holmes *et al.* 1985, Brannstrom & Flaherty 1995).

For perfusion, a volume- and pressure-controlled peristaltic pump (Storz Endomat n. Hamou; Karl Storz GmbH, Tuttlingen, Germany) was used. Flow was manually determined with a chronograph and a volumetric flask after 15 and 40 min, and then every 30 min. Pressure was continuously controlled by means of an instrument for invasive blood pressure measurement (Cardiocap 5; Datex-Ohmeda GE Healthcare Systems, Little Chalfont, England), and pressure in the ovarian artery (before entering the ovary) was calculated concurrent with flow measurements. Pressure was adjusted to achieve a perfusion flow of approximately 2 mL/min per gram ovarian tissue, as proposed (Stahler & Huch 1971). To avoid artefacts caused by accumulation of metabolites or hormones, Tyrode's solution was not recycled.

2.4. Study design

187 All ovaries were perfused for 240 min. During the first 60 min (equilibration), no agents
188 were added. In eight ovaries, 0.5 µg/mL *E. coli* O55:B5 lipopolysaccharide (LPS O55:B5
189 lyophilized powder; Sigma Aldrich, St. Louis, MO, USA) was added to the medium for
190 180 min after equilibration, whereas the other eight ovaries were not treated with LPS
191 throughout the entire experiment (control). For all ovaries, human chorionic gonadotropin
192 (hCG, 500 iu; Chorulon 1500®; MSD Animal Health GmbH, Luzern, Switzerland) was added
193 to the perfusion medium 210 min after the start of perfusion.
194
195 *2.5. Glucose, lactate, lactate dehydrogenase, creatine kinase, progesterone, and*
196 *prostaglandin $F_{2\alpha}$*
197
198 To ensure that the ovary remained in a physiological state, glucose and lactate
199 concentrations and activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were
200 used as markers of hypoxia and cell death (Richter *et al.* 2000).
201 Before its passage through the ovary, influent perfusion medium was sampled at 0, 60,
202 120, and 180 min after the start of perfusion. In addition, effluent perfusion medium was
203 sampled every 10 min throughout the entire perfusion period. At each time of sampling, three
204 native aliquots and one aliquot for glucose analysis (containing sodium fluoride as a
205 glycolysis inhibitor; Vacuette FX Sodium Fluoride / Potassium Oxalate; Greiner bio-one,
206 Kremsmünster, Austria) were collected and stored at -20 °C (P_4) or -80 °C (glucose, lactate,
207 LDH, CK, $PGF_{2\alpha}$), respectively.
208 Glucose concentrations were measured in all samples of influent perfusion medium. In
209 the effluent perfusion medium, concentrations of glucose and lactate, as well as LDH and CK
210 activities, were determined in samples collected after 10 min, and then every 30 min until the
211 end of perfusion. For these measurements, the Cobas Mira Plus analyzer (Roche, Basel,
212 Switzerland) with kits for glucose (Glucose RTU®; Biomérieux, Lyon, France), lactate

(Lactate PAP®; Biomérieux), LDH (LDH IFCC®; Axonlab), and CK (Enzyline CK NAC®; Biomérieux) was used. Analyses of glucose, lactate, LDH, and CK had a range of standard concentrations of 0.18 to 22.2 mmol/L, 0.04 to 10 mmol/L, 5 to 1200 U/L, and 5 to 1000 U/L, respectively. Intra- and inter-assay coefficients of variation were ≤0.92 and ≤2.55% for glucose, ≤1.14 and ≤3.20% for lactate, ≤1.14 and ≤1.41% for LDH, and ≤2.40 and ≤5.56% for CK, respectively.

Concentrations of P_4 were measured in the effluent perfusion medium every 10 min throughout the duration of perfusion, using a radioimmunoassay (RIA kit IM1188; Beckman Coulter GmbH, Krefeld, Germany). The range of standard concentrations for this test was 0.05 to 50 ng/mL, intra- and inter-assay coefficients of variation were ≤6.5 and ≤7.2%, respectively, and 50% of relative binding (ED50) occurred at 1.4 ng/mL.

Concentrations of $PGF_{2\alpha}$ were measured in effluent perfusate at 0, 10, 40, 70, 100, 130, 160, 190, 220, and 240 min of perfusion. A high-sensitivity $PGF_{2\alpha}$ ELISA kit (Enzo Life Sciences AG, Lausen, Switzerland) was used. For this test, the range of standard concentrations was 1.95 to 2000 pg/mL, intra- and inter-assay coefficients of variation were ≤7.2 and ≤11.0%, respectively, and ED50 was 81 pg/mL.

2.6. Corpus luteum biopsy and expression analysis

A biopsy (approximately 15 x 1 x 1 mm) was obtained from the maximum diameter (including cells from the periphery and the center) of the CL after 60, 120, 180, and 240 min of perfusion, using a semi-automatic, high-speed biopsy needle (TEMNO Evolution™; Fa. Walter, Baruth/Mark, Germany) that was rendered free of RNase (RNase-ExitusPlus™; AppliChem, Darmstadt, Germany). Tissue samples were immediately placed in a sterile DNase- and RNase-free cryo tube (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen (Dry Shipper Taylor-Wharton CX100; Jensons Scientific, Franklin, USA) and stored

239 at -80 °C until expression analysis was done. Immediately after the biopsy was collected,
240 perforation points on the CL surface were closed with fibrin glue (Histoacryl®; B. Braun
241 Melsungen AG, Melsungen, Germany).
242 Luteal mRNA expression was determined for luteinizing hormone / choriogonadotropin
243 receptor (*LHCGR*), steroidogenic acute regulatory protein (*STAR*), 3-beta-hydroxysteroid
244 dehydrogenase (*HSD3B*), *TLR2* and *-4*, caspase (*CASP*) *3*, *-8* and *-9*, prostaglandin E-
245 (*PGES/PTGES*) and -F (*PGFS/AKR1B1*) synthases, prostaglandin F receptor (*PTGFR/FP*),
246 *TNFA*, vascular endothelial growth factor A isoform 121 ($VEGFA_{121}$), fibroblast growth
247 factor 2 (*FGF2*), and endothelin 1 (*EDN1*). Therefore, total RNA from luteal tissue samples
248 was isolated and reverse-transcribed as described (Ulbrich *et al.* 2009) and luteal mRNA
249 expression was determined in a two-step quantitative real-time PCR (qPCR). The qPCR was
250 performed using the CFX384 Real-Time PCR Detection System (Bio-Rad, Munich,
251 Germany) and the SsoFast EvaGreen Supermix (Bio-Rad). The qPCR was performed in a
252 reaction volume of 10 µL, consisting of 5 µL EvaGreen Mix, 0.4 µL of each primer (10 µM),
253 0.07 µL VisiBlue (TATAA Biocenter, Göteborg, Sweden), 3.13 µL water, and 1 µL cDNA.
254 Primers used to amplify specific fragments referring to selected regulated genes are shown
255 (Table 1). Annealing temperature (AT) and melting points (MP) are outlined. The cycle
256 number (C_q) required to achieve a definite SYBR Green fluorescence signal was calculated by
257 the regression method (Bio-Rad CFX Manager 3.1). The C_q was inversely correlated to the
258 logarithm of the initial template concentration. The C_q determined for the target genes were
259 normalized against the geometrical mean of five reference genes (YWHAZ, H3F3A,
260 CNOT11, SUZ12, and TBP; ΔC_q). To avoid negative digits while allowing estimation of a
261 relative comparison between two genes, data were presented as means ± SEM added to the
262 arbitrary value 20 (ΔC_q). Thus, a high ΔC_q proportionally resembled high transcript
263 abundance (Livak & Schmittgen 2001).
264

265 *2.7. Postprocessing of ovaries*

266

267 After the 240 min study period, ovaries with *mesovarium* were weighed again to
268 estimate perfusion-induced edema. Subsequently, ovaries were perfused with stained (Patent
269 blue; Sigma-Aldrich) perfusion medium and dissected to test for smaller leakages and
270 homogenous staining of the CL. Ovaries with approximately more than one quarter of the
271 perfusion medium leaking from the ovarian artery before reaching the ovary, and/or with
272 poorly or non-homogenously stained CL were retrospectively excluded from the study.
273 Between each perfusion of an ovary, all glassware and tubing that were in contact with the
274 perfusion medium were cleaned and steam sterilized.

275

276 *2.8. Statistical analyses*

277

278 Data from only 11 ovaries were used; five ovaries (two from the control and three from
279 the LPS group) with leakages of the ovarian artery and non-homogenously perfused CL
280 (determined with Patent blue) were excluded. In excluded ovaries, the pressure in the ovarian
281 arteries decreased less ($P = 0.03$) during equilibration than in ovaries that were retained in the
282 study (22.8 ± 10.3 vs 44.8 ± 4.2 mmHg), indicating higher resistance, probably due to
283 intravascular coagulation. Consistently, mean P_4 concentrations during the complete perfusion
284 time were lower ($P < 0.0001$) in excluded (68.0 ± 6.4 ng/mL) than included ovaries (252.9 ±
285 23.5 ng/mL).

286 For included ovaries, the average interval between death of the cow and start of isolated
287 perfusion was 79.1 ± 3.2 min (mean ± SEM; range, 65 to 90 min), with no difference between
288 ovaries of the control (77.5 ± 4.6 min) and LPS groups (81.0 ± 4.6 min). Furthermore, the
289 ultrasonographically measured diameter of the CL was similar in control ovaries (27.8 ±
290 1.4 mm) and ovaries treated with LPS (27.5 ± 0.7 mm). Mean diameters and calculated cross-

sectional areas of the CL ranged from 25.5 to 33 mm and 5.11 to 8.55 cm^2, respectively, with the exception of only one CL with a diameter and area of 22.5 mm and 3.98 cm^2, respectively (for the latter CL, P_4 concentration in a blood sample collected immediately before slaughter was 7.2 ng/mL). Based on cross-sectional areas (n=10) or plasma P_4 concentration (n=1), CL were designated as mid-cycle (Days 8 to 16; Day 1 = ovulation) according to Herzog *et al.* (2010).

During equilibration (0 to 60 min after start of perfusion), pressure in the ovarian artery decreased by similar values in the control (42.1 ± 5.7 mmHg) and the LPS group (47.9 ± 6.6 mmHg), with no changes in arterial pressure during the treatment period (60 to 240 min after start of perfusion). The mean temperature and flow of the perfusion medium for all ovaries was 37.9 ± 0.1 °C (37.5 to 38.3 °C) and 37.7 ± 1.4 mL/min (29.3 to 44.5 mL/min), respectively. During the treatment period, there were no differences between control and LPS groups for temperature (38.2 ± 0.1 vs 38.2 ± 0.1 °C) or flow (38.0 ± 2.8 vs 36.5 ± 1.3 mL/min) of the perfusion medium.

In five cows of each group, the vascular pedicle started to contract frequently after a similar duration of perfusion (11.0 ± 1.0 min and 12.0 ± 2.6 min in the control and LPS groups, respectively). Total interval of frequent contractions did not differ between cows of the control (227.0 ± 2.0 min) and the LPS group (228.0 ± 2.6 min). During the last hour of perfusion, an intensification of contractions (not quantified) occurred in four ovaries of the LPS group, whereas contractions became less intense in the control group. The increase in weight of ovaries with vascular pedicle (due to edema in the *mesovarium*) did not differ between the control (30.3 ± 7.2 g) and the LPS group (47.1 ± 8.6 g).

Analysis of CK had a lower detection limit of 5 U/L. For measurements below this limit, 4 U/L was used as an arbitrary value to facilitate statistical analysis.

Distribution of the data was assessed visually for normality (PROC CHART) and by means of the Shapiro-Wilk-test (PROC UNIVARIATE). Because data were normally

distributed, independent (between groups; PROC TTEST) and dependent (within groups; PROC UNIVARIATE) pairwise comparisons were done using a Student's *t*-test, in due consideration of repeated measures. All statistical analyses were done with the Statistical Analysis System V9.1 (SAS Institute Inc., Cary, NC, USA), and $P \leq 0.05$ was considered significant.

3. Results

Mean glucose consumption during the perfusion time did not differ between control ovaries (mean ± SEM; 0.2 ± 0.03 mmol/L) and ovaries treated with LPS (0.2 ± 0.02 mmol/L). On a per-time basis, glucose consumption was higher (P = 0.03) in the LPS group compared to the control group at 180 min after the start of LPS challenge (Fig. 4A). Average lactate production during the perfusion period was not different between ovaries of the control (0.2 ± 0.04 mmol/L) and the LPS groups (0.2 ± 0.02 mmol/L), but on a per-time basis, lactate concentrations were increased 70 (P = 0.01) and 100 min (P = 0.03) after the start of treatment in ovaries of the LPS group compared to control ovaries (Fig. 4B). Mean enzyme activities of LDH and CK decreased (P < 0.05) during the equilibration time to nearly half of initial values, but thereafter remained relatively constant. For control and LPS ovaries, mean activities of LDH (12.3 ± 6.1 vs 7.3 ± 2.6 U/L, respectively) and CK (11.7 ± 2.3 vs 9.8 ± 1.1 U/L) were similar throughout the entire perfusion period. Furthermore, there was no difference in LDH and CK activities between these two groups on a per-time basis (Fig. 4C, D). In summary, parameters that characterized perfusion requirements (glucose consumption, lactate production, and activities of LDH and CK) were in a physiologic range at the start of the treatment period in both groups.

Progesterone concentrations in the effluent perfusate remained constant during the first 150 min of LPS treatment (mean ± SEM; 223.2 ± 41.3 ng/mL) and were similar to those of control ovaries (268.5 ± 35.0 ng/mL). However, after hCG challenge (150 min after the start of LPS treatment), P_4 concentrations increased in control, but not in LPS ovaries, and reached higher (P = 0.04) concentrations in control (402.4 ± 39.9 ng/mL) compared to LPS ovaries (241.9 ± 56.8 ng/mL) 30 min later (Fig. 5).

Mean concentrations of $PGF_{2\alpha}$ did not differ between the control (22.8 ± 6.9 pg/mL) and LPS groups (53.0 ± 18.5 pg/mL) throughout the entire treatment period. Furthermore,

348 there was no significant difference in $PGF_{2\alpha}$ concentrations between ovaries treated with LPS
349 and control ovaries on a per-day basis (Fig. 6) but $PGF_{2\alpha}$ concentrations tended (P = 0.10) to
350 be higher in LPS compared to control ovaries at 70 min after LPS challenge. After hCG
351 challenge, $PGF_{2\alpha}$ concentrations did not change within LPS and control ovaries.
352 Changes in mRNA expression of all investigated parameters are shown (Figure 7).
353 Luteal mRNA expression of steroidogenic factors *STAR* and *HSD3B* did not differ between
354 the control and the LPS group for any time of analysis (Fig. 7A). Furthermore, expression of
355 *LHCGR* did not differ between groups at any time (Fig. 7A). However, mRNA expression of
356 *TNFA* in the LPS group was higher at 60 (P = 0.0002) and 120 min (P = 0.004) after the start
357 of treatment compared to the control group (Fig. 7B). Furthermore, luteal expression of *TLR2*
358 was higher (P = 0.03) 180 min after the start of treatment in the LPS compared to the control
359 group, whereas expression of *TLR4* did not differ between groups at any time (Fig. 7B).
360 Analysis of apoptotic factors revealed higher mRNA expression in the LPS group for
361 *CASP3* at 60 (P = 0.02), 120 (P < 0.0001) and 180 min after start of treatment (P < 0.0001;
362 Fig. 7C). Higher expressions were also observed for *CASP8* at 60 (P = 0.05) and 180 min
363 (P = 0.01) after the start of LPS treatment compared to control ovaries, although *CASP9* did
364 not differ between groups at any time (Fig. 7C).
365 Whereas mRNA expressions of *PGES* and *PGFS* were higher in the LPS group at 120
366 (P = 0.008; only PGES) and 180 min (P = 0.03 and P = 0.05, respectively) compared to the
367 control group, expression of *PTGFR* did not differ between groups during the treatment
368 period (Fig. 7D). Luteal mRNA expression of angiogenic factor $VEGFA_{121}$ was higher
369 (P = 0.05) at 60 min after the start of treatment in the LPS compared to the control group
370 (Fig. 7E). In contrast, expressions of *FGF2* and *EDN1* did not differ at any time between the
371 control and the LPS group (Fig. 7E).
372

4. Discussion

In the present study, great efforts were made to maintain energy metabolism and viability of the ovaries similar to a physiological situation *in vivo* during the 3-h interval after equilibration. Therefore, the duration of ischemia was kept to a minimum, with an effort to maintain physiologic pressure in the ovarian artery, glucose consumption, lactate production, and LDH and CK activities to control vascular resistance (arterial pressure), oxygenation (glucose, lactate) and cytolytic tissue processes (LDH, CK), as suggested (Stahler & Huch 1971, Richter *et al.* 2000). Arterial perfusion pressure decreased markedly during equilibration but remained stable thereafter, indicating normalization of vascular resistance after re-perfusion. Furthermore, comparable metabolic activities in ovaries of the control and the LPS group were inferred, based on similar glucose consumption between groups. Lactate concentrations decreased directly after the start of perfusion in both groups. High lactate concentrations before perfusion were attributed to initial hypoxia (Ahren *et al.* 1972); therefore, re-perfusion with oxygenated medium decreased lactate production to physiologic values before the start of the treatment period. High concentrations of LDH and CK, as indicators of cell death during ischemia, decreased to a low and constant level within the equilibration period, consistent with a study on isolated perfused human uteri (Richter *et al.* 2000). In conclusion, there was good evidence that after equilibration, the general conditions were relatively physiologic, and therefore results obtained during the treatment period were reliable.

Increased lactate concentrations 70 min after the start of LPS challenge and the higher values in the LPS compared to the control group indicated increased metabolic activity, attributed to LPS-induced activation of immune defense mechanisms. Higher glucose uptake in the LPS group at the end of the perfusion period, which also indicated increased metabolic activity, was probably due to intensified contractions of the vascular pedicle in the LPS group

399 compared to the control group. Consistently, rhythmic contractions (2- to 3-min intervals) of
400 the vascular pedicle and the *hilus* area of the ovary were clearly visible in isolated perfused
401 sheep ovaries and the vascular pedicle was responsible for a non-negligible portion of glucose
402 uptake (Janson *et al.* 1978).

403 Luteal mRNA expression of *TNFA* was significantly higher in ovaries treated with LPS
404 than in control ovaries 60 and 120 min after the start of treatment. Consistently, LPS induces
405 TNFA production and the activated cytokine cascade (involving TNFA) mediates the acute
406 phase response to endotoxins (Kushibiki 2011). Functional TNF receptors were present in
407 steroidogenic and endothelial cells of bovine CL (Okuda *et al.* 1999, Okuda & Sakumoto
408 2003). Therefore, the ability of TNFA to modulate the lifespan of the bovine CL may largely
409 depend on the direct action on CL cells (Skarzynski *et al.* 2007). In cultured steroidogenic
410 luteal cells, TNFA inhibited gonadotropin-stimulated secretion (Benyo & Pate 1992),
411 consistent with failure of LPS-treated ovaries to increase P_4 concentrations after challenge
412 with hCG in the present study. Furthermore, TNFA acting *via* TNF receptor-1 induced
413 apoptotic death of steroidogenic and endothelial cells of the bovine CL through inactivation of
414 the anti-apoptotic protein Bcl-2 and by stimulating expression and activity of CASP3
415 (Skarzynski *et al.* 2005). Consistently, *CASP3* mRNA expression was increased 60, 120 and
416 180 min after the start of treatment in the LPS group compared to the control group.
417 Furthermore, mRNA expression of *CASP8* was increased 60 and 180 min after the start of
418 LPS treatment, whereas *CASP9* mRNA expression did not differ between LPS and control
419 groups, suggesting the importance of the extrinsic (death receptor mediated) pathway of
420 apoptosis.

421 Progesterone concentrations in the effluent perfusate did not significantly change during
422 the first 150 min after treatment with LPS (similar values in the two groups). In a previous
423 study on isolated perfused bovine ovaries (Sturm & Stahler 1971), P_4 concentrations in the
424 perfusate of untreated ovaries were 175 to 291 ng/mL, indicating adequate luteal release of P_4

425 in the present study. Previously, measurements of P_4 content in luteal tissue before and after
426 *in vitro* perfusion indicated that changes in P_4 concentrations of the effluent perfusate resulted
427 from differences in P_4 synthesis and release and not from leakage of P_4 already present in the
428 CL prior to perfusion (Dharmarajan *et al.* 1988). Furthermore, concentrations of P_4 in the
429 perfusate corresponded well with those synthesized in the tissue of CL from isolated perfused
430 ovaries (Brannstrom & Flaherty 1995), which were regarded as evidence of adequate
431 secretory capacity. However, our results were not consistent with an initial increase (within
432 30 min) and subsequent decline (until 9 h) of P_4 concentrations in an *in vivo* study involving
433 cows given LPS by intravenous treatment (Herzog *et al.* 2012). Notwithstanding, our results
434 supported the assertion that the initial increase in P_4 after treatment with LPS *in vivo* might be
435 of adrenal origin due to activation of a neuroendocrine stress axis (Kujjo *et al.* 1995, Herzog
436 *et al.* 2012).

437 Since LH or hCG significantly increase P_4 production in bovine midcycle CL (Koos *et*
438 *al.* 1984, Litch & Condon 1988), an hCG challenge was used in the present study to assess
439 secretory capacity of the CL during isolated perfusion. It was noteworthy that P_4 increased
440 significantly 30 min after application of hCG in the control, but not in the LPS group. In
441 cattle, LH binds to its specific receptor LHCGR and ultimately increases synthesis of STAR
442 and activity of cytochrome P450scc and HSD3B, followed by an increase in P_4 secretion
443 (Rekawiecki *et al.* 2005). However, in control ovaries in the present study, mRNA
444 expressions of *LHCGR*, *STAR* and *HSD3B* were not increased 30 min after hCG challenge,
445 although P_4 concentrations were already higher at that time. Consistently, concentrations of
446 mRNA for *STAR*, and *HSD3B* (measured at 3-h intervals) were not increased until 6 h after
447 luteal cell stimulation with LH (Rekawiecki *et al.* 2005). Furthermore, LH responsiveness
448 seemed to be more dependent on the extent of desensitization of luteal cells to LH (e.g., by
449 extensive clustering and internalization of the receptor complex) than on the number of LH
450 receptors, as during functional luteolysis, luteal cells apparently became desensitized to LH,

451 despite maintenance of LHCGR (Amsterdam *et al.* 2002). However, we inferred that the
452 absence of an increase in P_4 concentrations after LPS challenge was due to inhibited luteal
453 release rather than synthesis of P_4. Release of P_4 from the ovine CL was regulated by calcium-
454 dependent depolarization of the luteal cell membrane after stimulation with LH *in vitro*
455 (Higuchi *et al.* 1976).

456 Moreover, luteal $PGF_{2\alpha}$ concentrations in LPS-treated ovaries did not increase
457 significantly, although there was a trend towards significance. The release of $PGF_{2\alpha}$ from the
458 uterus is essential to suppress P_4 concentrations during luteolysis at the end of the estrous
459 cycle (McCracken *et al.* 1981). Whereas uterine $PGF_{2\alpha}$ has been clearly linked to luteolysis
460 (Schams & Berisha 2004), it is a debatable point whether luteal $PGF_{2\alpha}$ had a luteolytic effect
461 in the present study. However, changes in luteal mRNA expressions in ovaries of the LPS
462 group do not resemble changes observed after exogenous $PGF_{2\alpha}$ treatment in cows, including
463 decreased mRNA expressions of *PTGFR*, steroidogenic (*STAR*, *HSD3B*) and angiogenic
464 factors (*VEGF*, *FGF2*), and increased vasoactive factor *EDN1* mRNA expression (Shirasuna
465 *et al.* 2010). In the present study, there were no significant differences in these parameters
466 between control and LPS ovaries, except for a temporary increase in $VEGFA_{121}$, indicating a
467 non-specific response to LPS challenge. Therefore, we inferred that luteal $PGF_{2\alpha}$ was
468 probably not primarily responsible for LPS-induced changes in the present study.

469 Tumor necrosis factor alpha is a potent stimulator of luteal synthesis of prostaglandins
470 (including luteolytic $PGF_{2\alpha}$ and luteotropic PGE_2), consistent with increased mRNA
471 expressions of *PGES* at 120 and 180 min and *PGFS* at 180 min after the start of LPS
472 treatment compared to control ovaries, similar to a previous report (Okuda *et al.* 1999). In
473 bovine endometrial cells, PGE_2 and $PGF_{2\alpha}$ production were elevated after LPS challenge
474 (Herath *et al.* 2006), in association with extended luteal phases and premature regression of
475 the CL, respectively (Opsomer *et al.* 2000). The predominant effect (luteotropic or luteolytic)
476 was dependent on PGE_2 to $PGF_{2\alpha}$ ratio (Herath *et al.* 2006) and luteal phase (Shirasuna *et al.*

2010), with PGE_2 primarily controlling the early and $PGF_{2\alpha}$ the mid- and late luteal phases, respectively. In the present study using mid-luteal CL, increased mRNA expression of *PGFS* in the LPS group compared to the control group might have contributed to lower P_4 concentrations after hCG challenge in LPS-treated ovaries. Bovine *PGFS* mRNA encodes for 20α-hydroxysteroid dehydrogenase (20α-HSD; Madore *et al.* 2003, Schuler *et al.* 2006) that is responsible for P_4 catabolism within the CL (Naidansuren *et al.* 2011), and therefore might decrease P_4 in the effluent perfusate. Furthermore, luteal cells that express 20α-HSD not only lose their capacity to secrete P_4, but also facilitate expression of the death receptor Fas on their surface (Stocco *et al.* 2007), thereby enhancing apoptosis.

The main receptor for recognition of LPS is TLR4 (Akira 2003), whereas TLR2 predominantly recognizes peptidoglycans and lipoteichoic acid (Takeuchi *et al.* 1999). Expression of both receptors was reported in bovine ovaries (Vahanan *et al.* 2008). Interestingly, mRNA expression of *TLR4* did not differ significantly between control and LPS-treated ovaries in the present study, whereas expression of *TLR2* was higher in the LPS than in the control group 180 min after the start of treatment. Binding of the respective pathogens to TLR2 and -4 initiated a signaling cascade that resulted in release of TNFA and other cytokines (Kannaki *et al.* 2011). Since TNFA increased *TLR2* mRNA expression in various murine (Matsumura *et al.* 2000) and human (Davanian *et al.* 2012) tissues, an involvement of TNFA in higher mRNA abundance of *TLR2* after LPS challenge may have occurred in the present study.

In conclusion, treatment with LPS inhibited hCG-induced P_4 secretion in the bovine CL. The reduced P_4 secretion seemed to be predominantly caused by an increase in LPS-induced apoptosis, but not by decreased steroidogenic factors. Regardless, an impact of luteal $PGF_{2\alpha}$ could not be excluded.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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689

Figure legends

Fig. 1. *Mesovarium* stretched between the left ovary (A) and the left uterine horn (B) to display the ovarian artery (1) with its *ramus uterinus* (2) and *ramus tubarius* (3). Catheterization was performed at the marked position (→) proximally from the location where the *ramus uterinus* branched from the ovarian artery.

Fig. 2. Position of the ovary fixed at its *extremitates tubaria* and *uterina* on a bipod with the vascular pedicle (including the ovarian artery and vein) hanging downwards. The yellow color of the corpus luteum confirmed adequate preparation after flushing with heparinized Tyrode's solution.

Fig. 3. Schematic view of equipment used for isolated perfusion of bovine ovaries.

Fig. 4. Changes (means ± SEM) in glucose consumption (A), lactate concentration (B), and activities of lactate dehydrogenase (C) and creatine kinase (D) of control ovaries (○ ; n=6) and ovaries treated with LPS (● ; n=5) during equilibration and treatment times of perfusion. * Difference between the two groups ($P \leq 0.05$) at times indicated.

Fig. 5. Changes (means ± SEM) in progesterone concentrations of the effluent perfusate from control ovaries (○ ; n=6) and ovaries treated with LPS (● ; n=5) during the treatment period of perfusion.

→ Treatment with 500 iu hCG.

*Difference between the two groups ($P \leq 0.05$) at times indicated.

Fig. 6. Changes (means ± SEM) in prostaglandin $F_{2\alpha}$ concentrations of effluent perfusate from control ovaries (○ ; n=6) and ovaries treated with LPS (● ; n=5) during the treatment period of perfusion.

→ Treatment with 500 iu hCG.

Fig. 7. Changes (means ± SEM) in luteal mRNA expression of steroidogenic acute regulatory protein (*STAR*), 3-beta-hydroxysteroid dehydrogenase (*HSD3B*), luteinizing hormone / choriogonadotropin receptor (*LHCGR*), caspase (*CASP*) *3*, *CASP8*, *CASP9*, tumor necrosis factor alpha (*TNFA*), toll-like receptor (*TLR*) *2*, *TLR4*, prostaglandin E synthase (*PGES*), *PGFS*, prostaglandin F receptor (*PTGFR*), vascular endothelial growth factor A isoform 121 ($VEGFA_{121}$), fibroblast growth factor 2 (*FGF2*), and endothelin 1 (*EDN1*) of control ovaries (white columns; n=6) and ovaries treated with LPS (black columns; n=5) during the treatment period of perfusion.

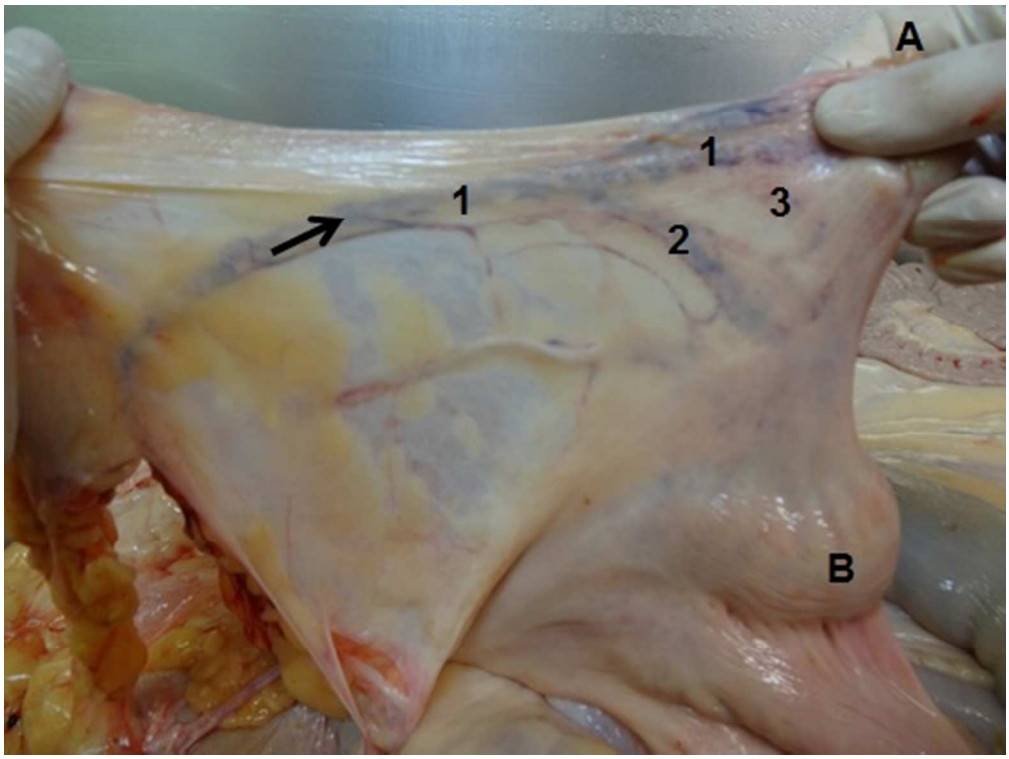
* Difference between groups ($P \leq 0.05$) at times indicated.

Table 1. Sequences and accession numbers of PCR primers for assayed genes from bovine corpus luteum cells, and length, annealing (AT) and melting point (MP) temperatures of PCR products.

Gene	Gene symbol	Reference	Forward primer	Reverse primer	PCR product	AT	MP
		[acc. no.]	[5'-…-3']	[5'-…-3']	[bp]	[°C]	[°C]
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	YWHAZ	NM_174814.2	AGGCTGAGCGATATGATGAC	GACCCTCCAAGATGACCTAC	141	60	81
histone	H3F3A	NM_001014389.2	ACTGGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	233	60	85
CCR4-NOT transcription complex, subunit 11	CNOT11	XM_582695.6	TCAGTGGACCAAAGCCACCTA	CTCCACACCGGTGCTGTTCT	170	60	87
suppressor of zeste 12 homolog (Drosophila)	SUZ12	NM_001205587.1	CATCCAAAAGGTGCTAGGATAGATG	TGGGCCTGCACACAAGAATG	160	60	82
TATA box binding protein	TBP	NM_001075742.1	CAGAGAGCTCCGGGATCGT	CACCATCTTCCCAGAACTGAATAT	194	60	83
luteinizing hormone / choriogonadotropin receptor	LHCGR	NM_174381.1	GGAAATGGATTTGAAGAAATACAA	GTGCTTTCACATTGTTTGGAAAAG	376	60	85
steroidogenic acute regulatory protein	STAR	NM_174189.2	GGATTAACCAGGTTCGGCG	CTCTCCTTCTTCCAGCCCTC	157	60	89
3-beta-hydroxysteroid dehydrogenase	HSD3B	NM_174343.3	TACCCAGCTGCTGTTGGA	ATGCCGTTGTTATTCAAGGC	322	60	87
toll-like receptor 2	TLR2	NM_174197.2	CCATGTGGAGAGGGTGTT	GGGGACACAAAACAGCACTT	138	60	81
toll-like receptor 4	TLR4	NM_174198.6	GACCCTTGCGTACAGGTTGT	GGTCCAGCATCTTGGTTGAT	103	60	80
caspase 3	CASP3	NM_001077840.1	AACCTCCGTGGATTCAAAATC	TTCAGGRTAATCCATTTTGTAAC[1]	114	60	80
caspase 8	CASP8	NM_001045970.2	TGTCACAATCGCTTCCAGAG	GAAGTTCAGGCACCTGCTTC	183	60	83
caspase 9	CASP9	NM_001205504.1	GTGGTGGAGAGCAGAAAGAC	AAACTAGACACGGCATCTGG	145	60	86
prostaglandin E synthase	PGES (PTGES)	NM_174443.2	TCCTGGTCTTCTTCCTGGG	CCCAGACAATCTGCAGGG	132	60	88
prostaglandin F synthase	PGFS (AKR1B1)	NM_001012519.1	ATACAAGCCGGCGGTTAAC	TGTCTGCAATCGCTTTGATC	188	60	86
prostaglandin F receptor	PTGFR (FP)	NM_181025.3	AGCCTTGCCATTGCTATCC	TAGTTCCATTGATGAGGTGCC	127	60	80

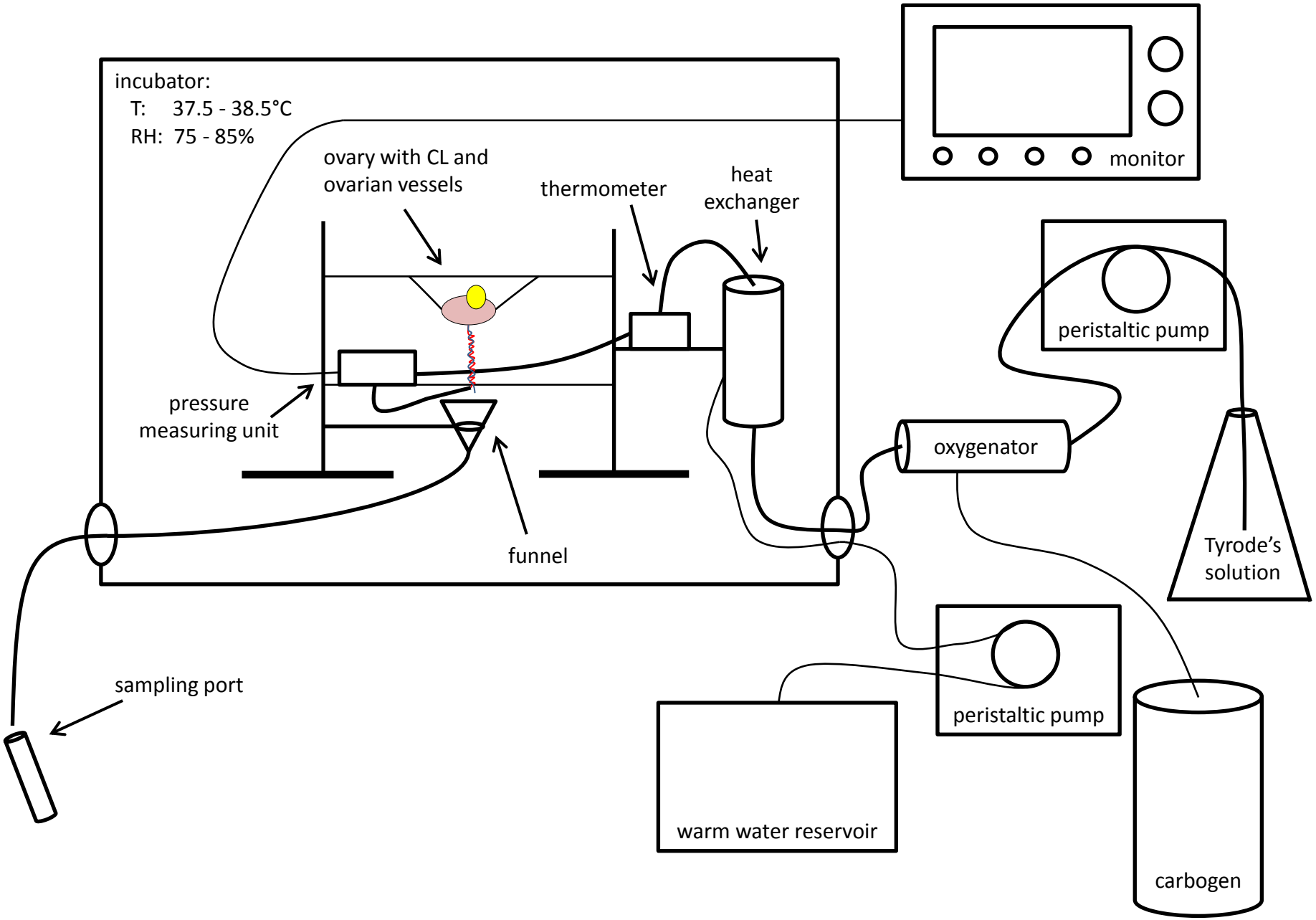
tumor necrosis factor alpha	TNFA	NM_173966.3	CCACGTTGTAGCCGACATC	ACCACCAGCTGGTTGTCTTC	108	60	86
vascular endothelial growth factor A isoform 121	$VEGFA_{121}$	NM_174216	CCGTCCCATTGAGACCCTG	CGGCTTGTCACAATTTTTCTTGTC	280	60	87
fibroblast growth factor 2 (basic)	FGF2	NM_174056.3	GAACGGGGGCTTCTTCCT	CCCAGTTCGTTTCAGTGCC	288	60	81
endothelin 1	EDN1	NM_181010.2	CTCTTCCCTGATGGATAAAGAGTG	GAACAACGTGCTCTGGAGTG	80	60	79

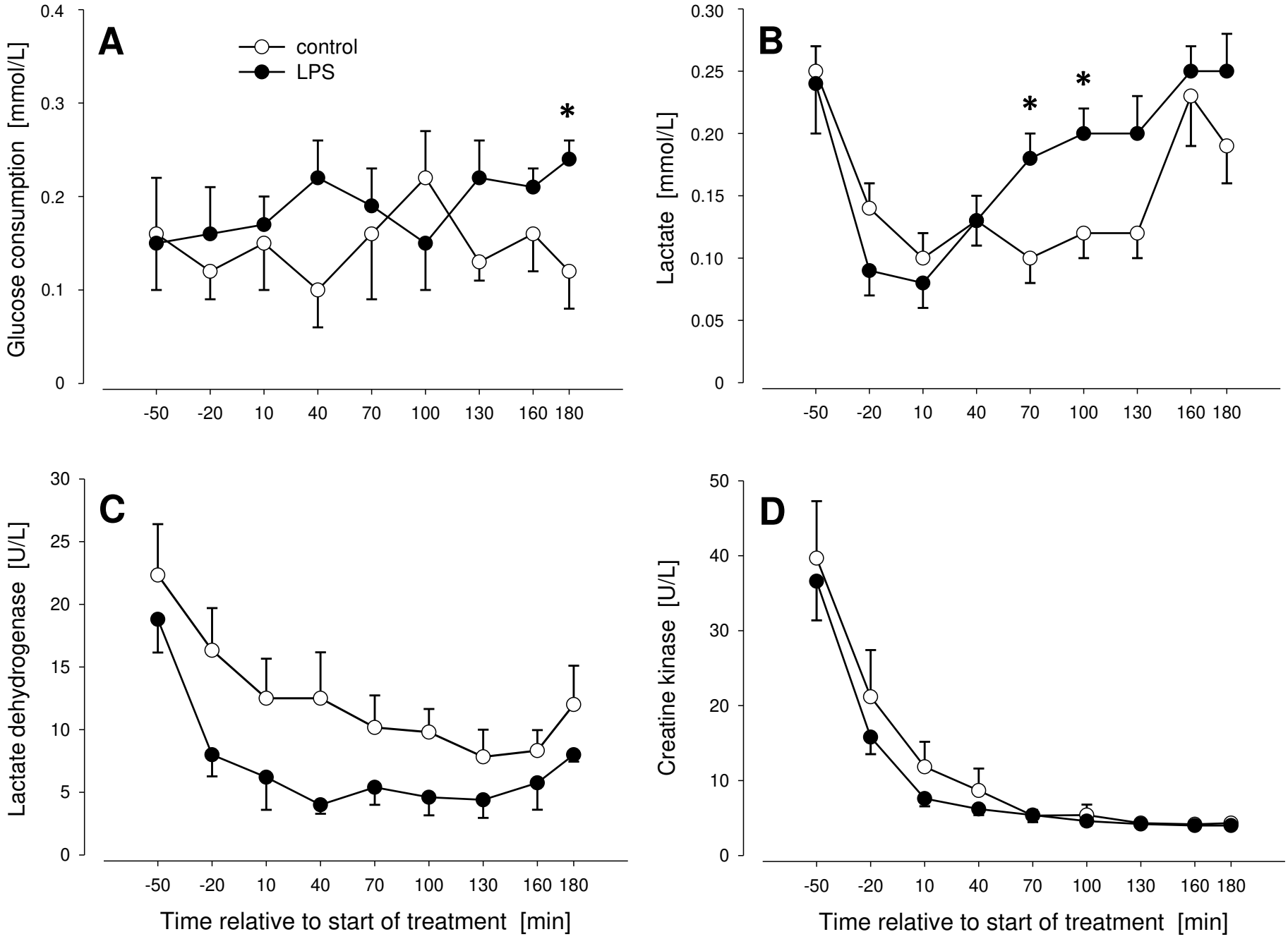
[1] degenerate multispecies primer, R = A or G

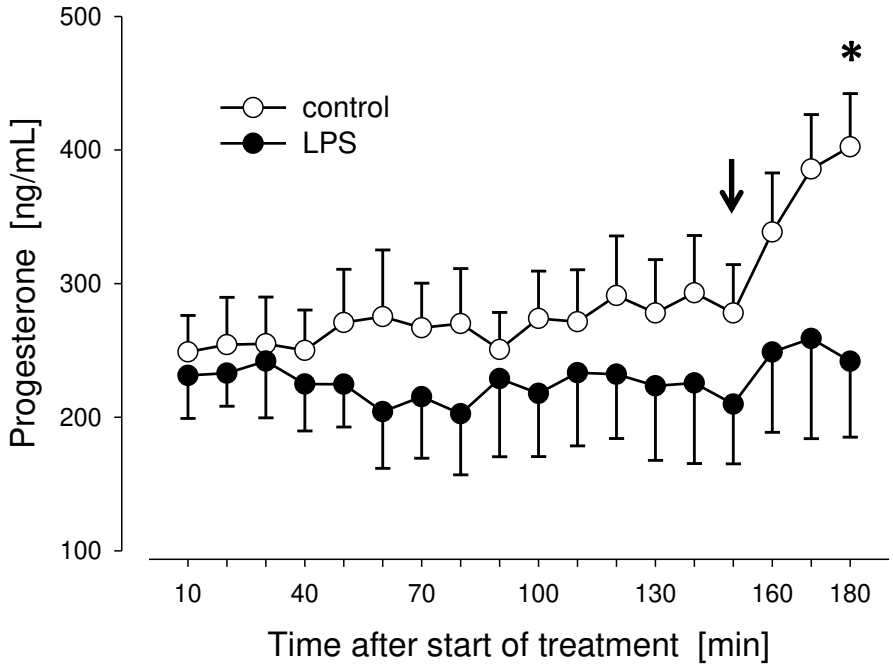


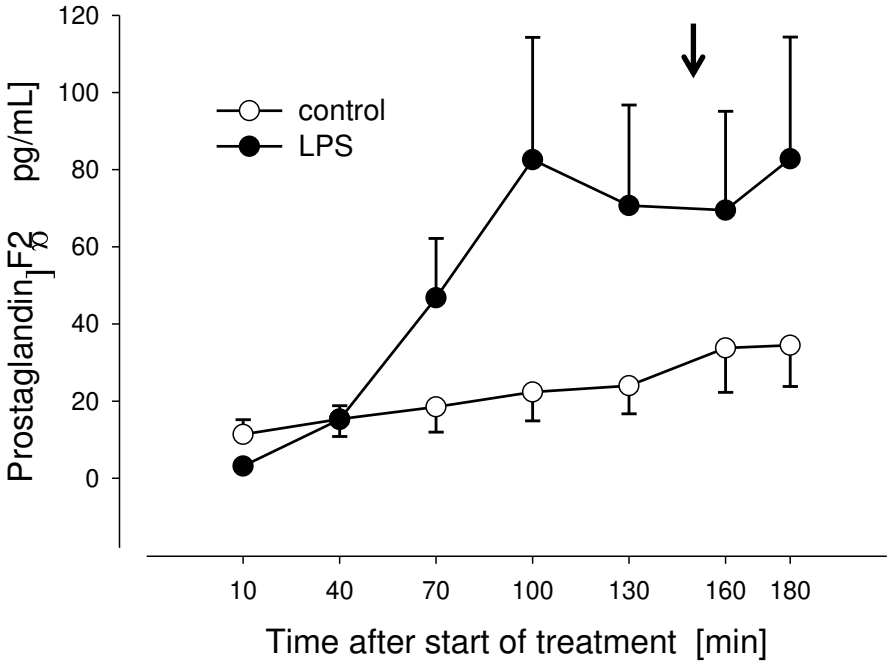
138x103mm (96 x 96 DPI)

115x87mm (96 x 96 DPI)

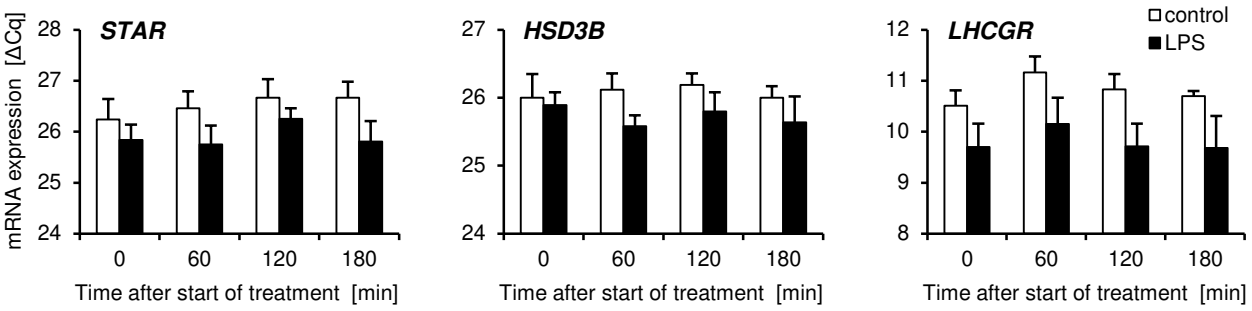

incubator:
T: 37.5 - 38.5°C
RH: 75 - 85%
ovary with CL and ovarian vessels
thermometer
heat exchanger
monitor
peristaltic pump
pressure measuring unit
oxygenator
Tyrode's solution
funnel
sampling port
peristaltic pump
warm water reservoir
carbogen


A
control
LPS
Glucose consumption [mmol/L]
0.4
0.3
0.2
0.1
0
*
-50 -20 10 40 70 100 130 160 180
B
Lactate [mmol/L]
0.30
0.25
0.20
0.15
0.10
0.05
0
*
*
-50 -20 10 40 70 100 130 160 180
C
Lactate dehydrogenase [U/L]
30
25
20
15
10
5
0
-50 -20 10 40 70 100 130 160 180
Time relative to start of treatment [min]
D
Creatine kinase [U/L]
50
40
30
20
10
0
-50 -20 10 40 70 100 130 160 180
Time relative to start of treatment [min]

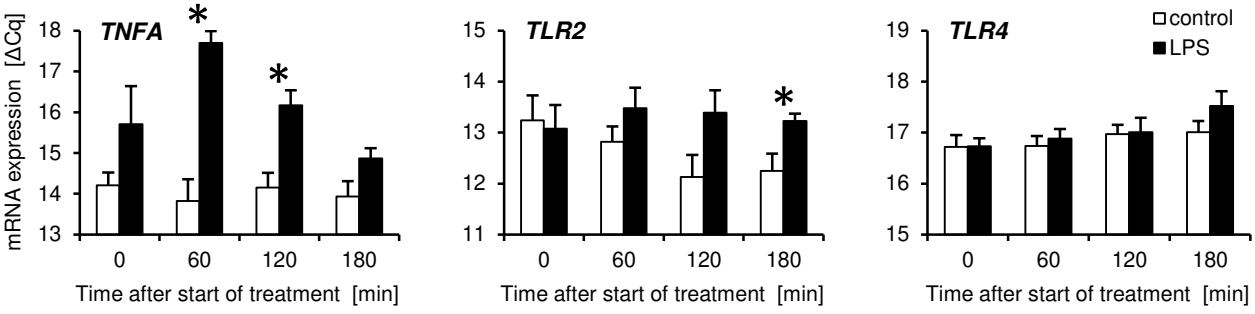

500
400
300
200
100
Progesterone [ng/mL]
control
LPS
*
10
40
70
100
130
160
180
Time after start of treatment [min]


120
100
80
60
40
20
0
Prostaglandin F2α [pg/mL]
control
LPS
10
40
70
100
130
160
180
Time after start of treatment [min]

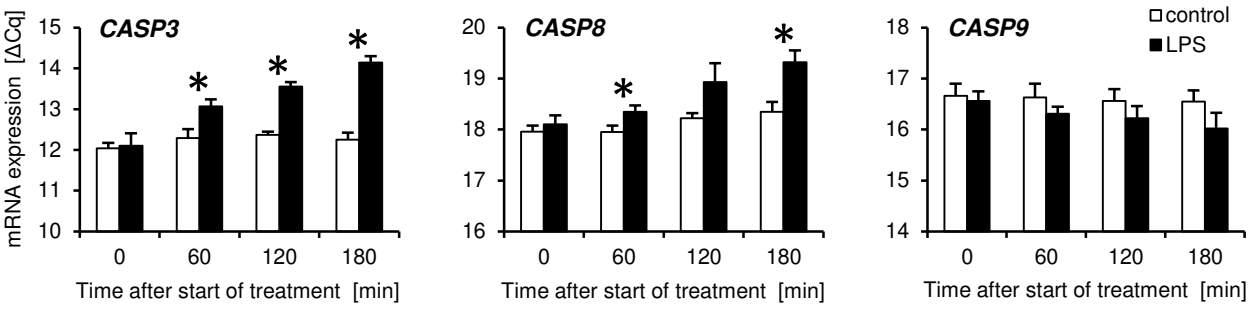
A) Steroidogenic factors and gonadotropin receptor



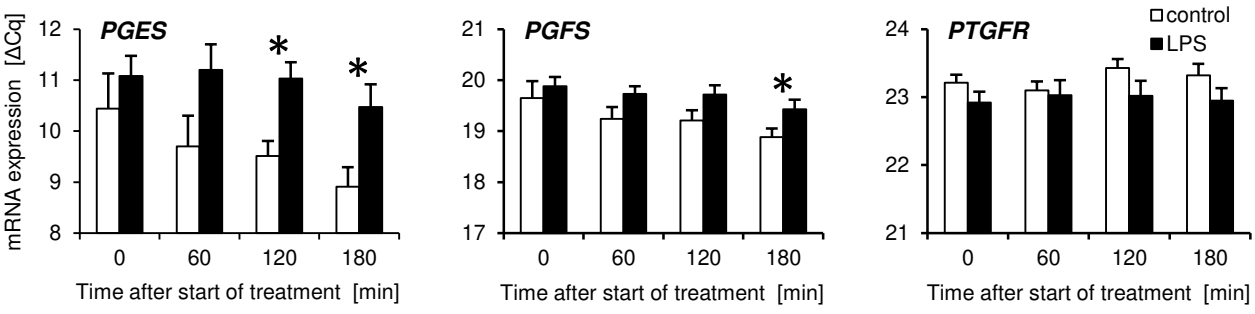
B) Proinflammatory cytokine and receptors for LPS



C) Apoptosis-related enzymes



D) Prostaglandin-related factors



E) Angiogenic and vasoactive factors